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Cholinergic Synaptic Signaling Mechanisms Underlying Behavioral Teratogenicity: Effects of Nicotine, Chlorpyrifos, and Heroin Converge on Protein Kinase C Translocation in the Intermedial Part of the Hyperstriatum Ventrale and on Imprinting Behavior in an Avian Model

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A wide variety of otherwise unrelated neuroteratogens elicit a common set of behavioral defects centering around cholinergic contributions to cognitive function. We utilized the developing chick to overcome confounds related to maternal effects and compared the actions of nicotine, chlorpyrifos, and heroin on cholinergic signaling in the intermedial part of the hyperstriatum ventrale (IMHV), which controls imprinting behavior. Chicken eggs were injected with nicotine (10 mg/kg of egg), chlorpyrifos (10 mg/kg of egg), or heroin (20 mg/kg of egg; all doses below the threshold for dysmorphology) on incubation days (ID) 0 and 5, and then tests were conducted posthatching. All three compounds elicited significant deficits in imprinting behavior. We also found defects in cholinergic synaptic signaling specifically involving the muscarinic receptor-mediated membrane translocation of protein kinase C (PKC)- γ and in the basal levels of both PKC γ and PKC β II, the two isoforms known to be relevant to behavioral performance. In contrast, there were no alterations in the response of PKC α , an isoform that does not contribute to the behavior, nor were cytosolic levels of any of the isoforms affected. Taken together with similar results obtained in rodents, our findings suggest that disparate neuroteratogens all involve signaling defects centering on the ability of cholinergic receptors to elicit PKC γ translocation/activation and that this effect is direct, i.e., not mediated by maternal confounds. The chick thus provides a suitable model for the rapid screening of neuroteratogens and elucidation of the mechanisms underlying behavioral anomalies.

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Key words: chick; IMHV; imprinting; PKC isoforms; teratogens

Exposure to various insults during prenatal development induces in humans and animals long lasting behavioral deficits (Yanai, 1984). To provide the groundwork for further studies on the reversal of the deficits, the focus of research recently is being directed toward the elucidation of the mechanisms by which the teratogen exerts its effect on behavior (Steingart et al., 2000b; Yanai et al., 2003). Despite the fact that neuroteratogens affect a variety of regions and innervations, one typical finding is cognitive impairment related to hippocampus-related behaviors and the associated cholinergic inputs (Yanai et al., 1992b). This provides both a conundrum and an opportunity. First, how can different compounds, with undoubtedly different originating mechanisms of action in the developing brain converge on a common final outcome? Second, if we can identify the synaptic pathways that are affected by disparate neuroteratogens, we may then be able to offset or reverse the defects. With these issues in mind, we studied prenatal exposure of mice to teratogens that act directly or indirectly on hippocampal cholinergic function (Yanai et al., 1992a; Shahak et al., 2003; Yanai and Yaniv, 2003; Yaniv et al., 2004) and identified a specific signaling

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defect that was common to all: interference with the ability of muscarinic cholinergic receptors to elicit membrane translocation/activation of protein kinase C (PKC)- γ (Ambalavanar et al., 1993; Shahak et al., 2003; Yanai et al., 2003; Yaniv et al., 2004). Ascertaining the locus and mechanism underlying the behavioral deficits enabled us to reverse both the synaptic and the cognitive dysfunction through therapies targeting septohippocampal cholinergic pathways (Yanai et al., 1992a; Steingart et al., 2000a).

However, the rodent model suffers from ineluctable methodological confounds stemming from drug effects on the maternal–fetal unit, maternal physiology, or mother–offspring interactions (Sastry, 1991; Sobrian et al., 1999). To mitigate these confounds, a species such as the chick is ideal: Drugs can be administered in defined doses without consideration of maternal–fetal or maternal–neonatal interactions, maternal toxicity, or pregnancy stage-related pharmacokinetic changes. Parallel to the hippocampus and its role in cognitive behaviors in mammals, avian species possess the left intermedial part of the hyperstriatum ventrale (IMHV), which is responsible for imprinting behavior (Bateson, 1966; Cipolla-Neto et al., 1982; Horn et al., 1983; Bradley et al., 1985; Bolhuis et al., 1989; Bradford and McCabe, 1994), the tendency of the chicks to follow the first object they encounter, which similarly involves cholinergic innervation (McCabe and Horn, 1991; Tsukada et al., 1999). Although imprinting normally involves the mother (Lorenz, 1935), it can readily be assessed with artificial objects (Bateson, 1966; Sluckin, 1972; Bolhuis et al., 2000). The IMHV contains a correspondingly high concentration of muscarinic cholinergic receptors (Bradley and Horn, 1981), and the release of acetylcholine in this region elicits the imprinting stimulus (McCabe and Horn, 1991; Tsukada et al., 1999). Just as for mammalian hippocampal cholinergic function, in the chick, PKC γ appears to play a pivotal role in the mechanism of imprinting behavior (Ambalavanar et al., 1993; Sheu et al., 1993; Van der Zee et al., 1995; Meberg et al., 1996).

In the current study, we evaluated whether muscarinic receptor-mediated translocation of behaviorally relevant PKC isoforms in the developing chick are targeted by the same disparate neuroteratogens that elicit those changes in the fetal mouse and whether the signaling defects are indeed accompanied by parallel deficits in imprinting behavior. We chose three compounds with differing primary mechanisms of action that nevertheless converge on septohippocampal function in the developing mouse, nicotine, chlorpyrifos, and heroin (Slotkin, 1999, 2004; Steingart et al., 2000b; Levin et al., 2002).

MATERIALS AND METHODS

Drug Treatments

Fertile heterogeneous stock eggs (60 ± 3 g) of the Cobb I chicken broiler strain (*Gallus gallus domesticus*) were obtained from a commercial source and placed in an incubator. To introduce substances, a hole was drilled in the chorioallantois

end (pointed end) of the shell 24 hr before the first injection and was covered with an adhesive silicone glue cap (Medical Type A; Dow Corning, Midland, MI). Drugs were then administered on incubation days 0 and 5, the period of time during which most of the brain structures, especially the IMHV, develop (Hamburger, 1951; Pearson, 1972). Control eggs received equivalent volumes (50 μ l/kg of egg) of corresponding vehicles: sodium tartrate for nicotine, dimethylsulfoxide (DMSO) for chlorpyrifos, and isotonic saline for heroin. We conducted preliminary studies on the effects of each of these vehicles compared with untreated eggs to ensure that none of them elicited developmental toxicity. In particular, DMSO, which is commonly used as a carrier for teratogens, is largely devoid of adverse effects (Hart et al., 1992; Kramer and Clem, 2003) unless very high doses are administered (Ferm, 1966); we were careful to use one to two orders of magnitude less DMSO than the amount required for teratogenic effects. Furthermore, in the present study, animals from the control groups receiving DMSO or sodium tartrate did not show any neurobehavioral differences from those receiving saline as a control for heroin.

Preliminary studies were conducted over a range of doses (nicotine, 2.5–20 mg/kg of egg; chlorpyrifos, 5–80 mg/kg of egg; heroin, 20–50 mg/kg of egg) to choose the optimal treatments that would produce behavioral defects without decreases in body weight or increases in leg deformities (spread legs). The main studies were then conducted at doses of 30 mg/kg of egg for nicotine bitartrate (Sigma Israel; equivalent to 10 mg/kg of nicotine free base), 10 mg/kg of egg for chlorpyrifos (kindly donated by Makhteshim Chemical Works, Israel), or 20 mg/kg of egg for heroin. Although the doses used in the present study for chicks are higher than the typical doses used for rodents (Roy et al., 2002; Garcia et al., 2003; Shahak et al., 2003; Yaniv et al., 2004), direct comparisons are not necessarily appropriate, given the differences in pharmacokinetics, dosing regimens (typically daily or multiple doses in rodents, only two doses for chicks), and the inherently lower sensitivity of chicks to the teratogens. The eggs were incubated (model 1202 incubator; G.Q.F. Manufacturing Co., Savannah, GA) at 37.5°C with 50–60% humidity and were candled on incubation days 5 and 15.

Fourteen to twenty-four hours posthatch, the chicks were trained to follow an imprinting object and were tested for imprinting performance. Afterward, the left IMHV was removed and taken for Western blot analysis of basal level of PKC isoforms and, for one of the agents (chlorpyrifos), for assessment of cholinergic receptor-mediated translocation/activation of PKC γ .

Induction and Testing of Imprinting

As modified from earlier descriptions by McCabe et al. (1981), the imprinting apparatus contained three 20-cm-diameter running wheels with the sides covered in black PVC, permitting the chicks to see only forward or backward. The imprinting objects were an illuminated red box or a blue cylinder (both 15 \times 10 \times 18 cm high), located 50 cm from the front open side of the running wheel, lit from within by a 40 W bulb with holes covered with red or blue filters, and rotated at 30 rpm. Imprinting training and testing were both assessed with this apparatus.

The imprinting procedure was modified from the one developed by McCabe et al. (1981). The chicks were hatched in total darkness and handling was done in the dark, aided by a dim green light, which has a minimal effect on imprinting (Kovach, 1971). All chicks were tagged and then transferred to an individual dark, enclosed wooden chamber warmed to 30°C, where they were physically and visually isolated from each other. Fourteen to twenty-four hours posthatch, the chicks underwent 45 min of "priming" and 30 min exposure to a light (60 W bulb), followed by 15 min of darkness. Immediately afterward, they were placed individually on the running wheel for training. The chicks were divided into groups trained for 60 min with either blue or red imprinting objects. The numbers of wheel rotations made by the chick toward or away from the imprinting object were recorded by a self-made computerized system. After training, the chicks were returned to the enclosed chambers for 60 min, after which testing took place. Recorded maternal calls were played continually throughout training but not during testing.

There were four testing sessions in counterbalanced, randomized order, each lasting for 5 min; in two of the tests, the chick was allowed to run toward the imprinting rotating object and, in the other two, toward the control rotating object. The red-light box was used as the imprinting object and the blue-light box served as the control object for chicks trained to follow a red object and vice versa for the chicks trained to follow a blue object. The numbers of wheel rotations completed by the chick toward the imprinting or the novel (control) object and the running away (backward) from the objects were recorded by the apparatus. The imprinting is expressed as preference scores where: Preference score = Running toward the training light / (Running toward the training light + Running toward a novel light). Running from the light is deducted from the score for running toward the light. The preference score is a measure of the strength of learning; assessing the selective preference that arises from the experience of the training object (Sluckin, 1972). The expected range of the preference score is 0.0–1.0, where 0.5 indicates no imprinting.

Locomotor Activity

Because locomotor activity influences the number of wheel rotations, we also assessed locomotor activity of the different experimental groups, as the number of rotations of the wheel made by the chick during training (forward and backward). The locomotor activity during imprinting testing (both training and novel lights) is expressed as the total number of rotations of the wheel (forward and backward) made by the chick.

Quantitative Assessment of PKC Isoforms and Translocation/Activation

After behavioral testing, the brain was removed, and the left IMHV (2.5–3.0 mg) was quickly dissected according to the procedure described by Horn (1991a,b) and frozen in liquid nitrogen, except for the carbachol-induced translocation/activation studies, in which fresh tissues were incubated and were frozen only after fractionation.

Basal levels of the PKC isoforms γ , β II, and α in the cytosolic and membrane fractions of the IMHV and the response

to incubation of tissue slices with or without the cholinergic agonist carbachol were assayed by using published protocols modified (Steingart et al., 2000b; Shahak et al., 2003; Yaniv et al., 2004) from earlier techniques (Towbin et al., 1979; Burnette, 1981). Accordingly, only brief descriptions will be provided here.

The IMHV tissues used for translocation/activation studies were sliced uniformly with a McIlwain tissue chopper, and the slices were then distributed equally in two separate tubes. Incubation took place in the presence or absence of 1 mM carbachol (carbamylcholine chloride; Sigma, St. Louis, MO) at 32°C for 0, 15, 30, or 40 min. After incubation, the tissues were washed twice with fresh buffer, homogenized, and then sedimented at 100,000g for 1 hr at 4°C, after which the supernatant solution containing the cytosolic fraction was frozen. The membrane pellet was resuspended and digested with 0.5% Triton X-100 (Sigma), then sedimented as described above, and the supernatant solution was frozen.

Western blot analysis was carried out by gel electrophoresis of 10–15- μ g aliquots of cytosolic and membrane protein with specific primary antibodies for each PKC subtype. The PKC γ antibody 36G9, which recognizes chick PKC γ (Van der Zee et al., 1995), was specially produced for this study (E.A. Van der Zee); cPKC β II and pPKC α were purchased from Santa Cruz Biochemicals (Santa Cruz, CA) and IgG HRP-conjugated (Bio-Rad, Hercules, CA) secondary antibody was used exactly as described earlier (Shahak et al., 2003; Yaniv et al., 2004). We did not assess internal standards of structural "housekeeping" proteins (α -tubulin or β -actin), because many neuroteratogens, including nicotine, chlorpyrifos, and heroin, influence neuro-morphological development (Eisch et al., 2000) and consequently cause alterations in the expression of these cytoskeleton proteins unrelated to the neurobehavioral effects linked to specific cell signaling pathways. Instead, to ensure standardization, samples with and without carbachol were assessed in slices from the same animal and were run in proximity to each other on the same gel. Because the response is assessed as the ratio of the value with carbachol to the value without carbachol, any inherent differences other than the specific concentration of PKC γ and PKC β II are equally represented in both the numerator and the denominator and do not contribute to the measured outcome. As an additional control, we evaluated PKC α isoform, an isozyme that does not participate in tasks such as spatial memory in the mouse and imprinting in the chick (Van der Zee et al., 1997; Colombo and Gallagher, 2002). A lack of corresponding alterations for PKC α ensures that any effect seen for PKC γ and PKC β II does not reflect artifacts in the Western blotting procedures.

Data Analysis

Data are presented as means and standard errors, with differences between treatments established by multivariate ANOVA, followed by the least significant difference test for post hoc comparisons between groups. The χ^2 test was employed for the nonparametric data. Significance for all tests was assumed at $P < 0.05$. For convenience, some results are presented as the percentage change from control values or as the percentage stimulation over basal PKC activity; however, statistical tests were always performed on the original data.

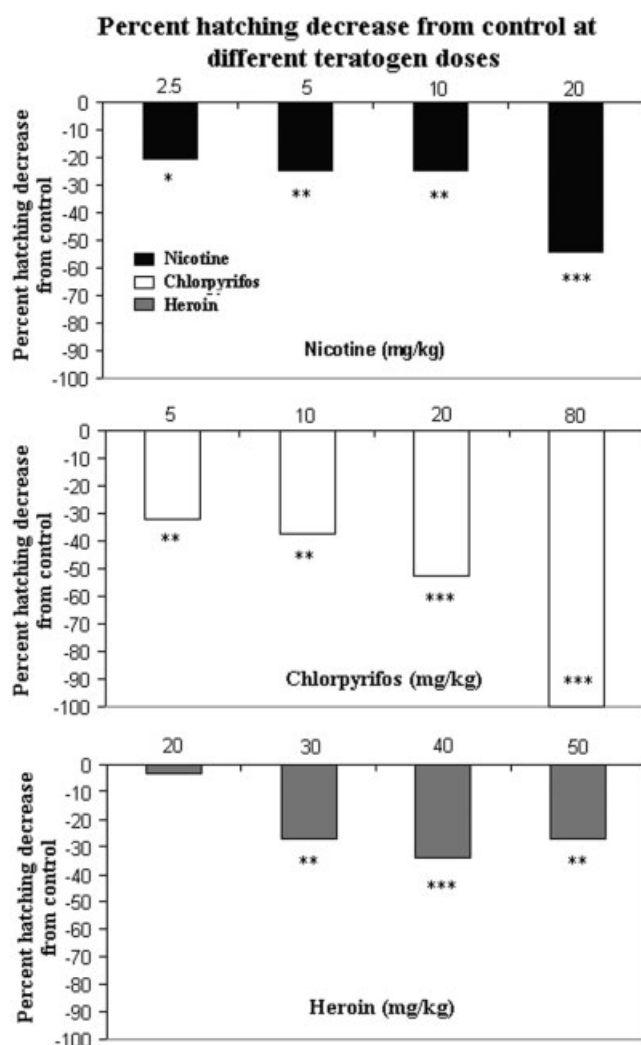


Fig. 1. Effect of prehatch exposure to different doses of nicotine, chlorpyrifos, or heroin (mg/kg of egg) on hatchability. Scores represent percentage hatching from control levels. Sample sizes were 250 eggs except for the following: chlorpyrifos 80 mg/kg of egg, $n = 100$; controls for the heroin group, $n = 516$; heroin 20 mg/kg of egg, $n = 470$; 30 mg/kg of egg, $n = 84$; heroin 40 mg/kg of egg, $n = 75$; heroin 50 mg/kg of egg, $n = 66$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, χ^2 test.

RESULTS

In our initial screening of developmental toxicity, we used a battery of standard evaluations to define a dose threshold for each agent at which to evaluate neurobehavioral effects. For nicotine and chlorpyrifos, reduced hatchability was evident at all doses, whereas, for heroin, the effect was noted only above 20 mg/kg of egg (Fig. 1). For the eggs that hatched, doses of 10 mg/kg of egg for nicotine or chlorpyrifos or 20 mg/kg for heroin were below the threshold for eliciting body weight deficits (Fig. 2); similarly, these doses did not increase the incidence of leg deformities (spread legs) above the 13%

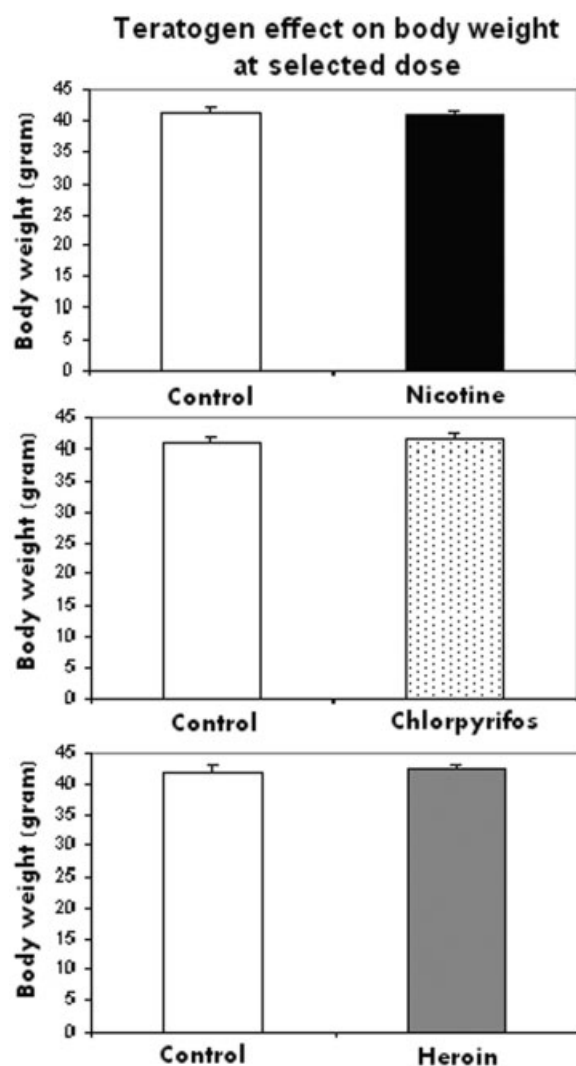


Fig. 2. Effect of prehatch exposure to different doses of nicotine, chlorpyrifos, or heroin on body weight (mean \pm SEM) among the hatched chicks from: 20 chicks in the control group for nicotine, 49 in the nicotine group, 25 in the control group for chlorpyrifos, 30 in the chlorpyrifos group, 12 in the control group for heroin, 23 in the heroin group. There were no statistically significant differences between groups (ANOVA).

incidence found in controls (data not shown). Accordingly, these doses were chosen for evaluations of behavioral performance or neurochemical effects; deformed chicks were excluded from the studies.

Imprinting preference scores among the three different control groups ranged from 0.78 to 0.82, well above the "no preference" score of 0.5 ($P < 0.001$). Prenatal exposure to nicotine or chlorpyrifos caused a robust decrease in imprinting, and heroin evoked a smaller, but statistically significant, decrease (Fig. 3). We evaluated general activity (number of rotations) to exclude potential confounding effects on activity in the imprinting evaluations (Fig. 4). Whereas nicotine or chlorpyrifos exposure had an effect on activity during train-

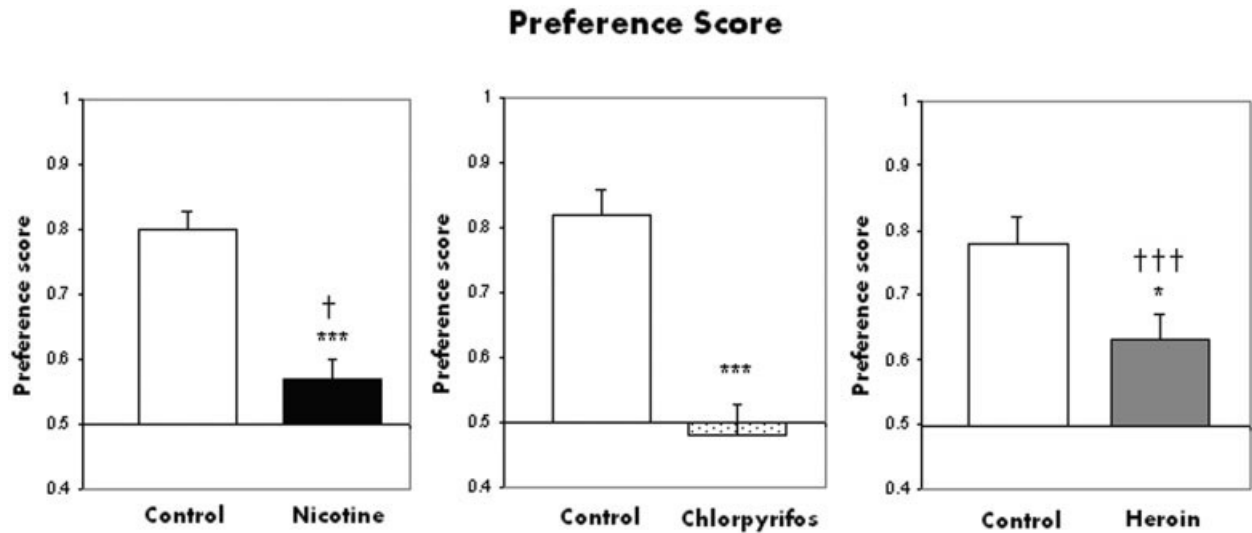


Fig. 3. Preference ratio in control chicks and chicks with prehatch exposure to nicotine, chlorpyrifos, or heroin. Data represent means \pm SEM obtained from 17 chicks in the control group for nicotine, 33 in the nicotine group, 23 in the control group for chlorpyrifos, 25 in the chlorpyrifos group, 11 in the control group for heroin, 21 in the heroin group. * $P < 0.05$, *** $P < 0.001$ vs. controls (ANOVA). † $P < 0.05$, ††† $P < 0.001$ for the difference from no preference (score = 0.5).

ing, these effects did not have a consistent pattern; nicotine *increased* and chlorpyrifos *decreased* general activity, and heroin had no effect. More importantly, during the testing period, prehatch exposure to the teratogens had no effect on general activity.

Each of the three neuroteratogens reduced membrane PKC γ in the IMHV by 15–25%, while eliciting no net change in cytosolic PKC γ (Fig. 5). Similarly, membrane-associated PKC β in the IMHV was significantly reduced by 20–30% without comparable reductions in cytosolic PKC β ; if anything, cytosolic activity was increased over control levels. In contrast to the γ and β isoforms, PKC α showed no deficits evoked by prenatal exposure to the teratogens, displaying only variable increases in cytosolic activity.

Before testing the effects of neuroteratogens on carbachol-induced PKC γ translocation/activation, we ran an experiment to establish the time course for optimal effect in chick IMHV preparations (Fig. 6A). The membrane PKC γ levels peaked at 15 min of incubation at 32°C, associated with a corresponding decrease in the cytosolic fraction. We then evaluated the response at 15 min in animals with prehatch exposure to chlorpyrifos (Fig. 6B). For membrane-associated PKC γ , control chicks showed a 68% increase in response to carbachol ($P < 0.04$), whereas there was no significant response in the chlorpyrifos group. In the cytosol, the control group again showed a decrease in PKC γ , although in this experiment the reduction did not reach statistical significance; there was no significant alteration in the chlorpyrifos group.

DISCUSSION

Prehatch exposure to subtoxic doses of nicotine, chlorpyrifos, and heroin, all teratogens that act directly or indirectly on the cholinergic innervation, induced marked deficits in the IMHV-related imprinting behavior and concomitant alterations in membrane PKC γ and β isoforms; PKC α , which is not implicated in learning-related behaviors, remained unchanged. Furthermore, the chlorpyrifos results indicate that the neuroteratogen interferes specifically with the muscarinic receptor-mediated translocation of PKC γ from the cytosol to the membrane, an event known to be required for the activation of this signaling molecule (Leaney et al., 2001).

Just as in the mammalian hippocampus, the chick provides a brain region- and innervation-specific model for neurobehavioral teratogenicity converging on cholinergic systems that regulate cognitive function. The essential difference is that, in the chick, the perturbations occur in a well-controlled environment, free of maternal confounds. In the chick, filial imprinting behavior, the tendency of the chicks to follow the first object they encounter, which is normally the mother (Lorenz, 1935), can also be assessed with artificial objects (Bateson, 1966; Sluckin, 1972; Bolhuis et al., 2000). Furthermore, avian species, unlike rodents, recognize color, so a variety of discrimination tasks based on the imprinting model can be carried out relatively simply. Imprinting depends on the integrity of a specific structure, the left side of the IMHV (McCabe et al., 1981; Bradford and McCabe, 1994), and the reliance of imprinting on this structure recapitulates the dependence of learning and memory in the mammalian hip-

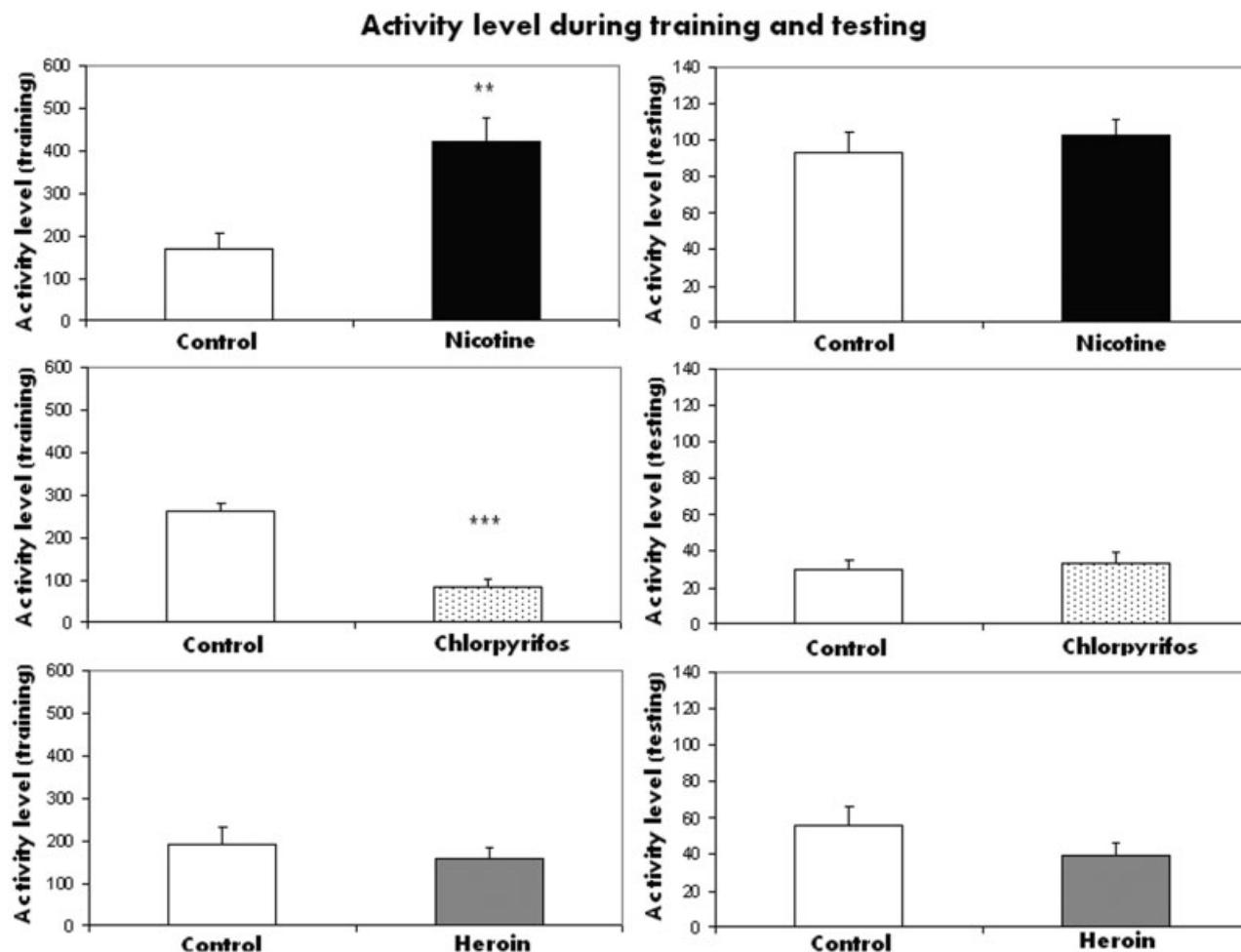


Fig. 4. Effect of prehatch exposure to teratogens on the activity level (wheel rotations) during training (left) and during testing (right). Data represent means and SEs obtained from the same chicks as for Figure 3. ** $P < 0.01$, *** $P < 0.001$ vs. controls.

pocampus (Kossut and Rose, 1984; Horn, 1991b, 1998), as established by biochemical, molecular, morphological, electrophysiological, and lesioning assessments (Bateson et al., 1969; McCabe et al., 1981; Bradford and McCabe, 1994). Furthermore, the relationship between synaptic function and behavioral endpoints is augmented by the fact that only the left IMHV stores the required imprinting information, whereas the right IMHV acts only as a temporary or "buffer" storage site (McCabe et al., 1981; Cipolla-Neto et al., 1982; Horn et al., 1983; Bradford and McCabe, 1994).

In addition to the avoidance of maternal confounds, the avian model allows true comparisons to be made of the relative impact of neuroteratogens on brain development compared with somatic growth, whereas mammalian models are highly dependent on potential adverse effects on maternal nutritional status, uteroplacental function, or endocrine changes elicited in the mother. Because the drug is delivered directly into the yolk sac, a strict rela-

tionship between dose and effect can be drawn in the absence of variables of maternal pharmacokinetics, which differ radically among species and with the stage of pregnancy. Moreover, whereas rodents will often kill or refuse to nurse neonates manifesting behavioral abnormalities, and require an extended period of postnatal development before cognitive function can be assessed, the chick is entirely self-sufficient, and as shown here, can be tested for cognitive function immediately after hatching. Unlike the case with the rodent, in which "litter effects" have to be taken into account (Spear and File, 1996), each chick is a separate subject, so that large numbers of animals can be assessed for high-throughput screening. In the present study, we demonstrated how the chick model allows rapid, simultaneous comparison of several different neuroteratogens. On the other hand, the chick model would be inappropriate for evaluation of neuroteratogens that require prior metabolic activation by the mother, that are excluded from the fetus by the placenta, or that share other

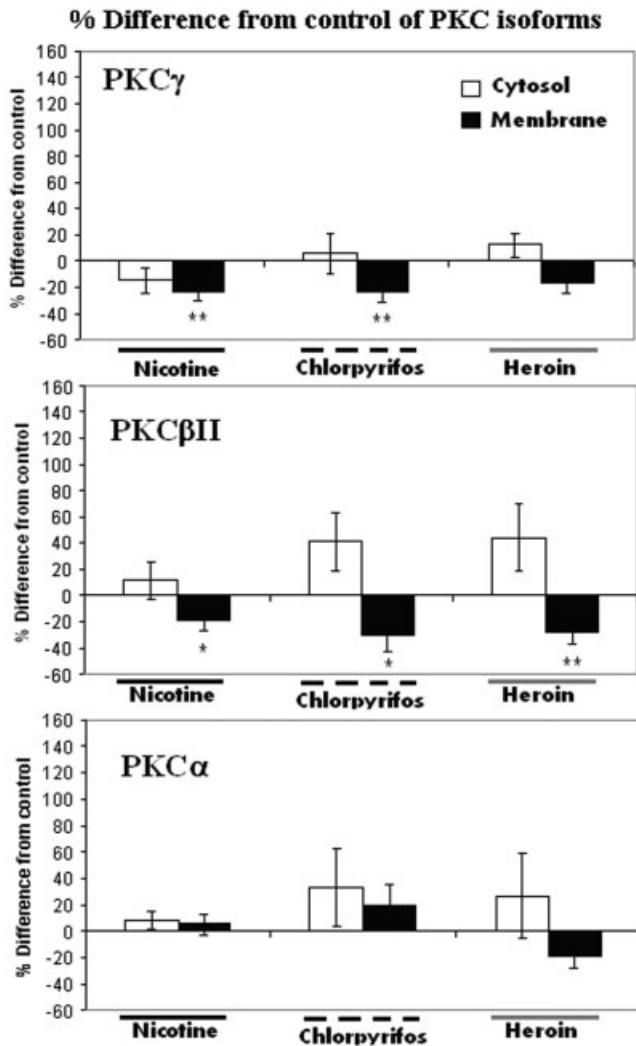


Fig. 5. Effect of prehatch exposure to nicotine, chlorpyrifos, or heroin on basal membrane and cytosolic levels of PKC isoforms. Data represent means and SEs of the differences from control levels. Group sizes are the same as in Figure 3. Basal control levels (OD) were: for cytosol, PKC γ , 0.53 ± 0.08 ; PKC β , 0.43 ± 0.10 ; and PKC α , 0.50 ± 0.07 ; the respective values for the membrane fraction are 0.52 ± 0.08 , 0.43 ± 0.10 , and 0.50 ± 0.07 . * $P < 0.05$, ** $P < 0.01$ vs. zero change.

attributes unique to mammals and not shared by avian species. Accordingly, although the chick model allows a high-throughput approach to neuroteratogen screening, the avian model obviously has lower stringency than a mammalian model, so that full evaluation of neuroteratogenic potential still requires comparative studies in mammalian species such as the rodent (Shahak et al., 2003; Yaniv et al., 2004). Given the large number of drugs and environmental contaminants that have to be evaluated for developmental neurotoxicity, a screening approach using the chick can help to focus mammalian studies on the compounds that are most likely to represent a liability.

Hatchability provides a prime example of endpoints that do *not* translate well from avian to mammalian species.

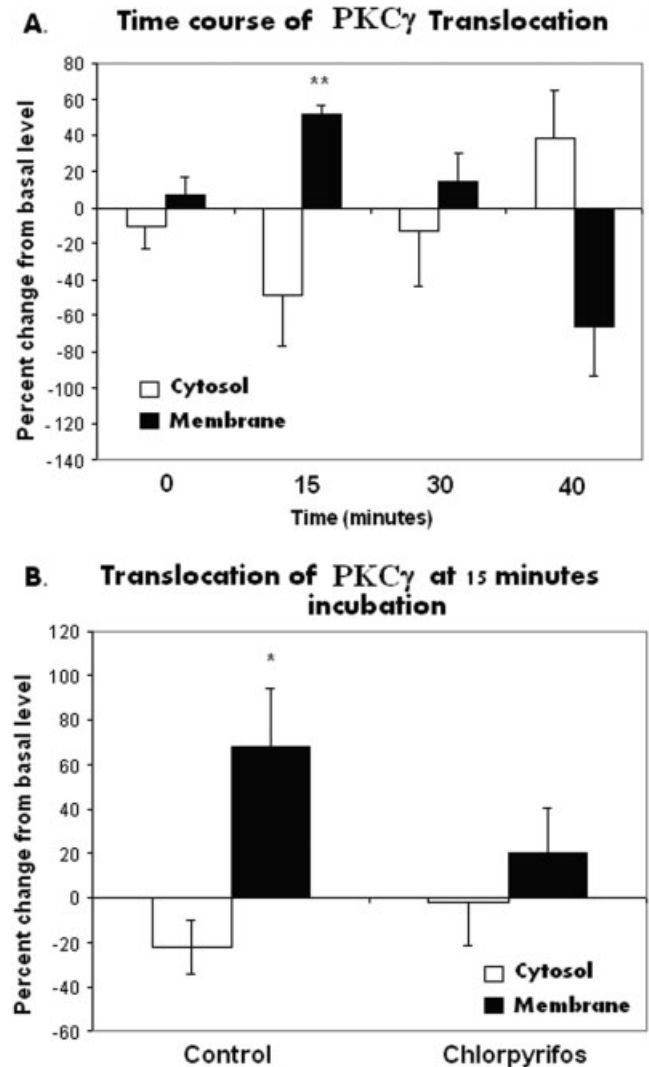


Fig. 6. **A:** Time course of the effect of carbachol on cytosolic and membrane PKC γ . Data represent means and SEs obtained from five homogenates in each group, with each sample comprising 10 chicks, except for the following: 15 min (cytosol) and 40 min (cytosol and membrane) obtained from four homogenates, with each sample comprising 10 chicks. Basal control levels (OD) were: PKC γ , cytosol, -0.43 ± 0.08 ; membrane, 0.81 ± 0.17 . ** $P < 0.01$ vs. zero change. **B:** Effect of prehatch exposure to chlorpyrifos on carbachol-induced (15 min) cytosolic and membrane PKC γ . Data represent means and SEs obtained from nine homogenates in each group, with each sample comprising 10 chicks, except for cytosol chlorpyrifos obtained from seven homogenates, with each sample comprising 10 chicks. Basal control levels (OD) are: PKC γ , cytosol -0.21 ± 0.09 ; membrane, 0.17 ± 0.05 . * $P < 0.05$ vs. zero change.

Nicotine, chlorpyrifos, and heroin all had significant effects on hatchability, and it would be tempting to draw parallels between this outcome and fetal resorption or spontaneous abortion in mammals. However, avian hatchability is extremely sensitive to otherwise inconsequential environmental perturbations, such as small changes in

temperature and humidity, seasonal variations, or clutch-to-clutch differences. Without any perturbation whatsoever, usually 10% of all eggs are infertile and an additional 10% will fail to hatch, and routine handling and/or saline injection reduce hatchability by an additional 15%. Whereas in mammalian species fetal growth retardation typically occurs at toxicant exposures below those that elicit fetal death, we found no effects on body weight in the chicks that hatched even at exposures reduced that reduced hatchability. Accordingly, whereas our results provide interpretable cross-species evaluations for neurobehavioral endpoints and for the relationship of these endpoints to malformations, the specific measure of hatchability is of limited utility in comparing benchmarks for toxicant exposures.

Because of the homologies between the rodent and the chick model in regional specificity, neurotransmitter pathways, and cognitive outcomes after exposure to neuroteratogens, avian species are likely to provide valuable information about synaptic mechanisms that underlie behavioral deficits. In the current study, we explored how different neuroteratogens that compromise cholinergic function converge on a common downstream defect in the signaling protein PKC, mainly involving the γ isoform. Nicotine, heroin, and chlorpyrifos all operate through different originating mechanisms, yet, in the present study, the final effects on imprinting and the related cholinergic synaptic function showed convergent effects. Nicotine affects brain development through hyperstimulation of nicotinic cholinergic receptors, thus disrupting the trophic role of acetylcholine in brain development (Slotkin, 1999, 2004). Chlorpyrifos similarly evokes cholinergic stimulation through inhibition of cholinesterase but also elicits direct actions on signaling and transcription factors involved in neural cell differentiation (Slotkin, 1999, 2004). Heroin acts on opioidergic innervation, which in turn affects the development of cholinergic function indirectly (Yanai et al., 1992a; Steingart et al., 2000a); accordingly, we found that the more directly acting cholinergic agents, nicotine and chlorpyrifos, had a relatively greater effect on imprinting than did heroin. In conjunction with the cholinergic deficits caused by each of these agents, the present study found common interference with basal levels of PKC γ and PKC β and with the membrane translocation/activation in response to cholinergic stimulation, consistent with their known involvement in learning and memory (Sheu et al., 1993), and for PKC γ , in imprinting (Ambalavanar et al., 1993; Sheu et al., 1993). For imprinting, PKC β is not implicated directly but is associated with contextual fear conditioning (Phillips and LeDoux, 1992; Abeliovich et al., 1993), and overcoming fear of the novel imprinting object is likely to represent an important contributor to imprinting ability. Consistent with previous studies in rodent models (Shahak et al., 2003; Yaniv et al., 2004), PKC α , which is not implicated in learning-related behaviors, was also unaffected by the cholinergic teratogens in the chick model, further supporting the hypothesis that only specific PKC isoforms are relevant to the deficits in cholinergic function and cognitive performance.

Beyond the biologic implications of our findings, there are important methodologic considerations that will influence future studies. First, evaluation of PKC isoforms by Western blot analysis provides an assessment only of the total number of molecules, without telling us about their *function*. This is particularly true for PKC γ (Shahak et al., 2003; Yaniv et al., 2004), for which a phosphospecific antibody is not yet available for any species, and also in the chick for PKC β . Even if the antibodies were available, these would be unable to characterize the specific cholinergic-receptor-mediated component of translocation/activation as distinct from the more general pool of enzyme. Accordingly, the techniques used here are likely to prove more generally applicable in identifying defects in cellular function centering around neurotransmitter control of the PKC signaling cascade. Furthermore, there are clear species differences in the cellular events involved in PKC translocation/activation. In our earlier work with the mouse, there were two peaks of PKC γ translocation/activation, representing a fast (15 min) and a slow (40 min) wave of activity (Shahak et al., 2003; Yaniv et al., 2004). The time course for translocation/activation in the chick showed only the fast wave (Yaniv et al., 2004), suggesting the absence of some PKC-related cellular events in the avian species. Future work should address the biologic significance of these temporal differences in the cholinergic response.

In conclusion, the present study establishes a model for the screening of neuroteratogens and also for the elucidation of the synaptic mechanisms that connect specific neural defects to adverse behavioral outcomes of disparate neuroteratogens. The identification of specific cellular defects that represent the downstream common pathways mediating cognitive impairment after neuroteratogen exposure will allow future research to focus on the development of interventions that can reverse neuroteratogenicity.

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